Robust monooxygenase biocatalysts

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Chapter 2:

Baeyer-Villiger Monooxygenases: Tunable Biocatalysts for Oxidative Chemistry

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Abstract

Pollution, accidents, and misinformation have earned the pharmaceutical and chemical industry a poor public reputation, despite their undisputable importance to society. Biotechnological advances hold the promise to enable a future of drastically reduced environmental impact and rigorously more efficient production routes at the same time. This is exemplified in the Baeyer-Villiger reaction, which offers a simple synthetic route to oxidize ketones to esters, but application is hampered by the requirement of hazardous and dangerous reagents. As an attractive alternative, flavin-containing Baeyer-Villiger monooxygenases (BVMOs) have been investigated for their potential as biocatalysts for a long time, and many variants have been characterized. After a general look at the state of biotechnology, we here summarize the literature on biochemical characterizations, mechanistic and structural investigations, as well as enzyme engineering efforts in BVMOs. With a focus on recent developments, we critically outline the advances towards tuning enzymes suitable for industrial applications.

Introduction

"The field of organic chemistry is exhausted."[1] This notion, which many scientists later judged a fallacy,[2] was not an isolated opinion in the late 19th century[3] from when the quote stems. It is ascribed to chemist Adolf von Baeyer and supposedly was a reaction to the success in synthesizing glucose,[4] achieved by his earlier student, Emil Fischer. While Fischer was said to share von Baeyer's confidence,[3] their potential rush to judgment did not prevent either of them to later be awarded the Nobel Prize. In the wake of ever more discoveries being made, scientists today largely refrain from such drastically exclusivistic statements and rather call organic chemistry a 'mature science'.[5]
In hindsight, the time of von Baeyer's controversial statement can in fact be considered as the early days of organic synthesis. Chemistry only started to transform from an analytic to a synthetic discipline after 1828,[6] when Wöhler's urea synthesis was the first proof that organic compounds don't require a 'vital force'.[7] Similarly to this paradigm shift in chemistry nearly 200 years ago, biology is currently at a turning point.[6,8] Although bread making and beer-brewing can be considered biotechnological processes invented thousands of years ago, the deliberate creation of synthetic biological systems only succeeded in the late 20th century. As much of modern research, biotechnology is an interdisciplinary area,[5] but a particularly strong overlap with organic synthesis occurs in the field of biocatalysis. One of the main arguments for using enzymes for chemical transformations is that even if inventions in organic chemistry will never exhaust—its major feedstock soon will. Considering the continuing depletion of the world's fossil fuel reserves, a major contemporary challenge represents the switch to synthetic routes starting from alternative building blocks. In the light of the chemical industry and their supplier's historically disastrous impact on the environment,[9] a second challenge is the transition to what has been termed "green chemistry":[10] the choice of building blocks from sustainable sources and the avoidance of hazardous substances. Moreover, with the chemical industry being the single most energy intensive industry sector worldwide,[11] strategies to increase efficiency of chemical processes are urgently needed. Unfortunately, however, such considerations find only reluctant implementation in practice. Despite an increased public pressure due to the poor reputation of the chemical industry,[12] the market economy still nearly irrevocably ensures the design of industrial processes by economical considerations.[13] In research, delaying factors might include the hesitancy to rethink traditional approaches and the fact that environmental considerations are often inconspicuous on lab-scale, or out of focus due to the limited scientific prestige.[12-13] In the meantime, biocatalytic transformations emerged as a profoundly different alternative.

Besides the prospect of inherently green catalysts, a hallmark of biocatalysis is product selectivity, as enzymatic reactions arguably allow total control over chemo-, regio-, and enantioselectivity. This renders biocatalysis especially useful for the preparation of pharmaceuticals, where the isomeric impurity can have dramatic physiological consequences.[14] One of the biggest assets of enzymes is the prospective of their targeted functional evolvability.[15-16] Ever more sophisticated molecular biological methods for DNA manipulation allow easy access to large numbers of enzyme variants, which can be screened for desired activities. Despite being one of the oldest techniques, random mutagenesis libraries continues to be an extremely successful enzyme engineering approach. On the other hand, more rational approaches guided by structural and biochemical data in combination with computational predictions have gained popularity.[17] Although still impractical in most scenarios, the complete *de novo* design of enzymes has been demonstrated and likely will become a key technology in the future.[18]

Although often seen as a limitation, the usually found restriction of enzymes to aqueous systems and ambient temperatures is also advantageous: these processes not only abide by the principles of green chemistry, the consistency in process conditions also facilitates the design of cascade reactions, which circumvents the need to isolate intermediate products. Cascades can be designed as *in vitro* processes, in which chemoenzymatic strategies may combine the power of chemo- and biocatalysis.[19] With whole cells as catalysts being the economically most attractive approach, another highly promising procedure is to establish cascades fully *in vivo*. Recent advances in genetic manipulation techniques greatly accelerated metabolic engineering approaches, allowing the introduction of foreign metabolic pathways into recombinant microbial hosts. These pathways may be of natural origin, partially adapted, or designed entirely *de novo*. Recent examples of the recombinant production of natural products such as opiods[20-21] or cannabinoids[22] attracted considerable attention not only in the scientific community. Artificial metabolic routes designed in a "bioretrosynthetic"[23] fashion, on the other hand, allow diverse applications ranging from novel CO_2 fixation strategies[24] to the production of synthetic compounds such as the anti-malarial drug artemisinin.[25] With research in this area of biotechnology rapidly developing, it has been suggested to constitute a new field called synthetic metabolism.[26]

The Baeyer-Villiger reaction of peroxides and monooxygenases

Presumably, considerations of green chemistry were far from the mind of the before-mentioned Adolf von Baeyer, when 110 years ago, he and his disciple

Victor Villiger were experimenting with potassium monopersulfate. In the honor of their discovery that this and other peroxides can oxidize ketones to esters, we now call this the Baeyer-Villiger reaction. Although it is a widely known method in organic chemistry nowadays,[27-28] several unsolved difficulties reduce its attractiveness and thus applicability. Especially on large scale, a remaining problem is the shock-sensitivity and explosiveness of many peroxides.[29] Commonly applied peracids are prepared from their corresponding acids using concentrated hydrogen peroxide. As these solutions in high concentrations are prone to ignition and other forms of violent decomposition,[30] they have largely been withdrawn from the market.[31] Reactions with peroxides and peracids furthermore lead to stoichiometric amounts of hazardous waste products. More promise lies in recent achievements of reactions using hydrogen peroxide as the oxidant, which make use of metal or organocatalysts. However, such processes also require waste treatment and the catalysts need to be prepared in additional, often complex synthetic routes.

Due to these reasons, biocatalysis offers a particularly promising alternative and has attracted considerable attention. So-called Baeyer-Villiger monooxygenases (BVMOs) use the free, abundant, and green oxidant O_2, and only generate water as byproduct. BVMOs were discovered in the late 1960s by Forney and Markovetz, who were interested in the microbial catabolism of naturally occurring, long-chain methyl ketones. They noticed that the products generated from these compounds by a *Pseudomonad* were incompatible with terminal methyl oxidation, which was the previously assumed only degradation pathway.[32] Subsequently, they were able to identify the responsible enzymatic reaction as a Baeyer-Villiger transformation, dependent on NADPH and molecular oxygen.[33] In parallel, Trudgill and coworkers were investigating microorganisms able to grow on non-naturally occurring aliphatics. They identified an oxygen and NADPH-dependent enzyme from *Acinetobacter calcoaceticus* NCIMB 9871 involved in the microbial metabolism of fossil fuel-derived cyclohexane and suggested that it catalyzes the conversion of cyclohexanone to ε-caprolactone.[34] They confirmed their findings by isolating the protein and established that the enzyme contains a flavin adenine dinucleotide cofactor as prosthetic group.[35] This cyclohexanone monooxygenase (AcCHMO) quickly attracted attention because of its broad substrate scope and because caprolactone was already well-known as a precursor to nylon 6.[36-37]

Sequences and structures

In the last decades, many BVMOs, both prokaryotic and eukaryotic, have been described and approximately a hundred representatives were cloned and recombinantly expressed. In many cases, the natural role of those BVMOs could not be identified. In other cases, BVMOs were shown to be involved in the biosynthesis of secondary metabolites such as toxins,[38-40] or antibiotics.[41] While these enzymes seem to be more frequently rather substrate specific, several BVMOs from catabolic pathways, involved e.g. in the degradation of cyclic aliphatics,[34,42-44] can convert a large range of substrates. Together with the structurally very similar N-hydroxylating- and flavin-containing monooxygenases, BVMOs have been classified as belonging to the class B of flavoprotein monooxygenases.[45] Recently added to this class are the YUCCAs[46]—plant enzymes involved in auxin biosynthesis that were shown to catalyze a Baeyer-Villiger-like reaction.[47] Some FMOs, including the human isoform 5,[48] were also found to catalyze Baeyer-Villiger reactions.[49] This subgroup was suggested to be classified as class II FMOs[50] and their relaxed coenzyme specificity[51] enables interesting application opportunities.[52] Structurally largely unrelated are a few Baeyer-Villiger reaction-catalyzing enzymes found in class A[53] and C,[54] which otherwise comprise the aromatic hydroxylases and luciferases, respectively.[45] Cytochrome P450 monooxygenases, which also sometimes catalyze Baeyer-Villiger reactions,[55-56] are entirely unrelated and employ heme cofactors instead of flavins.

Considerable research has been performed on BVMOs using comparative sequence analysis. Using a curated, representative sequence set, one study suggested that a BVMO gene was already present in the last universal common ancestor.[57] This study also found that there is no conclusive evidence that phylogenetic BVMO subgroups share biocatalytic properties, although this frequently has been and continues to be suggested in literature.[58-60] Several residues in BVMOs are highly conserved,[61] and besides containing two GxGxx[G/A]Rossmann fold motifs required for tight cofactor binding,[62] they can be identified by two fingerprint motifs: FxGxxxHxxxW[P/D] and [A/G]GxWxxxx[F/Y]P[G/M]xxxD.[50,63] The exact functional role of the fingerprint residues has remained unclear and also the determination of BVMO's three-dimensional structure could not clarify their strict conservation. The first crystal structure was solved for phenylacetone monooxygenase (PAMO) from *Thermobifida fusca*;[64] since then, seven other enzyme and various mutant structures followed (Table 1), totaling to 38 structures at the time of writing.

Table 1. Available BVMO crystal structures

Name	Acronym	Source strain	Uniprot ID	PDB entries	Ref.
cyclohexanone monooxygenase	AcCHMO	*Acinetobacter calcoaceticus NCIMB9871*	Q9R2F5	6A37[a]	65
Aspergillus flavus monooxygenase 838	Af838MO	*Aspergillus flavus* NRRL3357	B8N653	5J7X	66
cyclohexanone monooxygenase	RhCHMO	*Rhodococcus* sp. HI-31	C0STX7	3GWD, 3GWF, 3UCL, 4RG3, 4RG4	67-69
cyclohexanone monooxygenase	RpCHMO	*Rhodococcus* sp. Phi1	Q84H73	6ERA[a], 6ER9	70
cyclohexanone monooxygenase	TmCHMO	*Thermocrispum municipale* DSM 44069	A0A1L1QK39	5M10, 5M0Z, 6GQI	71-72
2-oxo-Δ^3–4,5,5-trimethylcyclopentenylacetyl-coenzyme A monooxygenase	OTEMO	*Pseudomonas putida*	H3JQW0	3UOV, 3UOX, 3UOY, 3UOZ, 3UP4, 3UP5	73
phenylacetone monooxygenase	PAMO	*Thermobifida fusca* YX	Q47PU3	1W4X, 2YLR, 2YLS, 2YLT, 2YLW[a], 2YLX[a], 2YLZ[a], 2YM1[a], 2YM2[a], 4C74, 4C77[a], 4D03[a], 4D04[a], 4OVI	64,74-75
Parvibaculum lavamentivoran monooxygenase	PlBVMO	*Parvibaculum lavamentivorans*	A7HU16	6JDK	76
polycyclic ketone monooxygenase	PockeMO	*Thermothelomyces thermophila* ATCC 42464	G2QA95	5MQ6	59
steroid monooxygenase	STMO	*Rhodococcus rhodochrous*	O50641	4AOS, 4AOX, 4AP1[a],4AP3[a]	77

[a]Mutated variant

Mechanistic insights have mostly been gained by structural studies on CHMO from *Rhodococcus* sp. HI-31 (RhCHMO) and PAMO. Overall, the structures of BVMOs are surprising similar, despite sequence similarities of often less than 40%. With the exception of PAMO, many BVMOs are often rather unstable; however, no obvious structural features could be identified as the origin of this stability. However, one study compared PAMO and AcCHMO's tolerance towards cosolvents—a feature frequently shown to be related to thermostability.[78] The authors suggest that an increased number of ionic bridges in PAMO caused the lower susceptibility to solvents, thus preventing damage to the secondary and tertiary structure.[79] The same reasoning was given for the higher robustness of a recently crystalized CHMO from

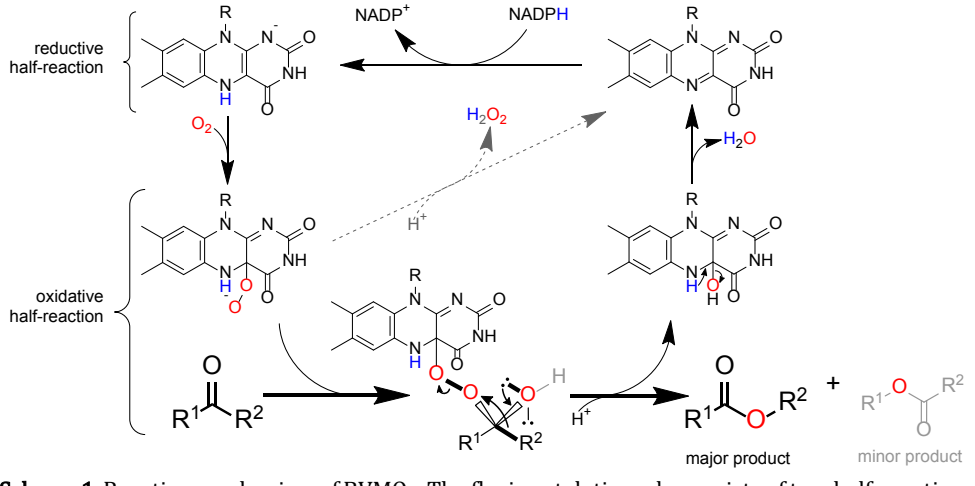
Thermocrispum municipale (TmCHMO).[71] BVMOs display a multi-domain architecture consisting of an FAD-binding, an NADP-binding and a helical domain. The latter distinguishes BVMOs from other class B flavoprotein monooxygenase families and causes the formation of a tunnel towards the active site, which it partially shields. Some BVMO subgroups contain N-terminal extensions of varying length. The structure of such an extension was established in PockeMO, where it forms a long helix and several loops that wrap around the enzyme.[59] This enzyme is more thermostable than most BVMOs, but it is unknown whether the extension plays a role in that. Such a function was suggested for 4-hydroxyacetophenone monooxygenase (HAPMO), where deletion of the extension was not tolerated when exceeding a few amino acids.[80] Removal of only nine amino acids already impaired stability and furthermore decreased the enzyme's tendency to dimerize. Besides FAD, which is found in all BVMO crystal structures, the nicotinamide cofactor is also bound in many structures, in accordance with its tight binding to the enzymes.[45]

Mechanism of the Baeyer-Villiger reaction

Catalysis is initiated by NADPH binding and subsequent flavin reduction, after which the nicotinamide cofactor adopts a stable position.[67,75] Flavoproteins allow detailed mechanistic studies due to the characteristic absorption spectra traversed by the flavin cofactor during the various states of catalysis (Scheme 1). In BVMOs, a stable peroxyflavin was identified to be the catalytically active species.[81] Formed by the radical reaction[82] of two electron-reduced FAD with molecular oxygen, this spectroscopically observable flavin intermediate was already known from the flavin-dependent aromatic hydroxylases[83] and luciferases.[84] The finding was perhaps rather unsurprising, considering that the chemical Baeyer-Villiger reaction is also afforded by peroxides. However, while with few exceptions,[28] the chemical reaction is acid catalyzed, thus entailing a protonated peroxide, the catalytic flavin species requires a deprotonated peroxy group.[85] While quickly decaying in solution,[86] some BVMOs stabilize this reactive species for several minutes in the absence of a substrate, before its decomposition forms hydrogen peroxide in the "uncoupling" side reaction known from all monooxygenases.[87-90] Two factors are critical for this stabilization: an arginine residue in the active site of the enzyme, whose mutation abolishes Baeyer-Villiger activity,[91] and $NADP^+$, which establishes a hydrogen bond to the hydrogen of the flavin's N5 and thus prevents uncoupling.[92] If a suitable ketone substrate is available, the next canonical step is the nucleophilic attack on the carbonyl group. In BVMOs, the proper positioning of the substrate is thought to be aided by a hydrogen bond

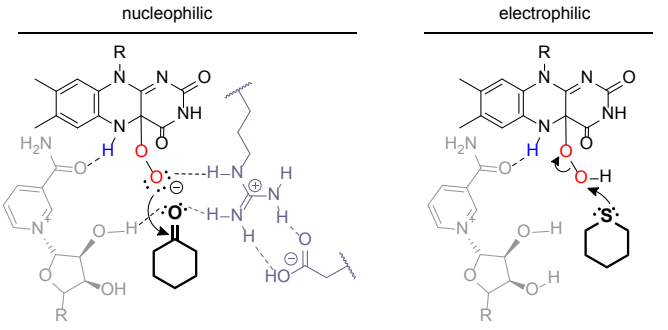
between the 2' OH group of the $NADP^+$ ribose and the carbonyl oxygen (Scheme 2).[93] The chemical reaction was already for a long time assumed to proceed via an intermediate whose nature initially caused some debate. Isotopic labeling experiments[94] eventually gave conclusive evidence for the pathway suggested by Rudolf Criegee,[95] in whose honor the tetrahedral intermediate was subsequently named. Although not directly observable, several computational studies support this mechanism.[96-99] Very recently, experimental evidence was provided from a stereoelectronic trap for the intermediate using synthetic endocyclic peroxylactones.[100] Also a flavin Criegee intermediate was never observed, but in the absence of any counter-evidence it is generally accepted that the flavin and substrate in the BVMO reaction also form an addition product, and computational studies support this theory.[93,101] The product is then formed in a concerted subsequent migration step, in which the weak O–O bond is heterolytically cleaved, while a new C–O bond is formed. The rearrangement proceeds under retention of configuration[102-103] and is often rate-determining, although both experimental[28] and theoretical[96] evidence indicate that the kinetics can change depending on the substituents, pH, and solvent. The regiomeric outcome of the reaction is generally predictable and governed by a combination of influencing parameters. Firstly, due to the positive charge developing on the migrating carbon in the transition state, the more electronegative carbon, which is better able to accommodate this charge, is more apt to migrate.[104] Thus, carbons with electron donating substituents and those allowing resonance stabilization migrate better than methyl groups and electron withdrawing substituents.[28] Secondly, the C–C bond migrates preferentially when it is *anti*-periplanar to the peroxy O–O bond (Scheme 1), a condition known as the primary stereoelectronic effect.[105] Its influence on determining migration is apparently more significant than the migratory aptitude. This was concluded from the observation that a less substituted bond migrates when forced into an *anti*-periplanar conformation in a restrained bicyclic Criegee intermediate.[106] A secondary stereoelectronic effect has also been postulated, requiring that one of the lone electron pairs of the hydroxyl group in the intermediate also needs to be *anti*-periplanar to the peroxy O–O bond (Scheme 1).[107] This effect only manifests in certain substrates, where substituents can sterically hinder the hydroxyl group rotation and presumably plays no role in enzyme catalysis, where the hydroxyl group is assumed to be deprotonated.[93] Lastly, the arrangement can be influenced by steric effects.[108-109] These may furthermore already affect the addition step, where the nucleophilic attack must occur from a favorable angle.[28,110] Steric control becomes most obvious in the enzymatic reaction, where intermolecular steric restraints can enforce an otherwise

electronically prohibited pathway. It is for that reason that BVMO catalysis allows the synthesis of products, which are not accessible by chemical means. While the peroxide-catalyzed reaction finishes under formation of the corresponding acid, the flavin can pick up a proton to form a hydroxyflavin, whose spontaneous dehydration reconstitutes the oxidized flavin.[86] It was suggested that this step is accelerated by a deprotonated active site residue, in line with the faster decay of this species at higher pH and the decreased overall reaction rates at low pH.[85,111] Before the enzyme can restart a new catalytic cycle, the oxidized nicotinamide cofactor needs to be ejected, and this step (or an associated conformational change) was found to be limiting to the overall reaction rate in CHMO.[85] In PAMO, the slowest catalytic step was not unambiguously identifiable, but may correspond to a conformational change prior to $NADP^+$ release.[111] Considering the complexity of the various effects taking place in the transformation, a generalization on the mechanism for all BVMOs and substrates may not be possible. If it was, however, a general rule that the rate-determining step in enzyme catalysis is substrate-independent, it could provide an explanation for the rather narrow range of maximal turnover rates observed for BVMOs with various substrates.



Scheme 1. Reaction mechanism of BVMOs. The flavin catalytic cycle consists of two half-reactions and ketone oxidation is catalyzed by a peroxyflavin, unless hydrogen peroxide loss causes an uncoupled $NADP^+$ oxidation (grey dashed arrow). The transformation from a ketone to an ester traverses through a regioselectivity-determining intermediate. Bond migration is dependent on the *anti*-periplanar alignment (indicated by thick bonds) of the migrating bond with the peroxy bond and one of the lone pairs on the former carbonyl oxygen. While protonated in the chemical Baeyer-Villiger reaction, this oxygen is, however, thought to be deprotonated in enzyme flavin intermediate (indicated in grey).

Promiscuous catalytic activities

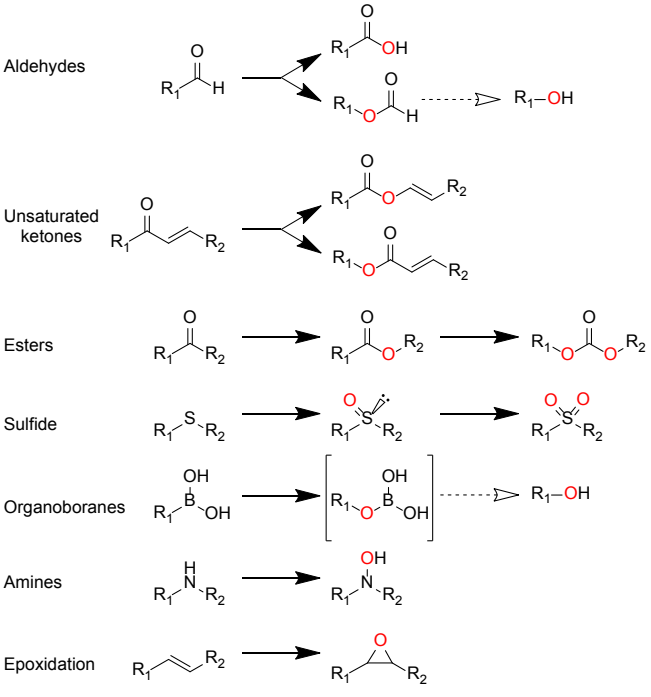


Scheme 2. Proposed mechanism for enzyme catalyzed oxidations. In the canonical, nucleophilic mechanism, the peroxyflavin attacks the substrate carbonyl. An active site aspartate increases the basicity of a neighboring arginine, which thus ensures deprotonation of the peroxyflavin. The arginine also activates the substrate ketone, supported by the 2' OH of the ribose of $NADP^+$. In contrast, in the electrophilic mechanism a supposed hydroperoxyflavin reacts with the lone pair of a nucleophilic heteroatom.

Well-established and mechanistically analogous to the canonical reaction, are BVMO oxidations of aldehydes,[112-117] including furans.[118] This reaction yields acids upon hydrogen migration, or otherwise (often unstable) formates. Although reactions with unsaturated ketones supposedly should also proceed identical in mechanism, most BVMOs show no reactivity with these poorer electrophiles. The transformation is also challenging chemically, where side reactions such as epoxidations frequently occur, and otherwise invariably enol esters are formed, i.e. oxygen insertion occurs towards the double bond.[119] Recently, two bacterial BVMOs were reported that can convert several cyclic α,β-unsaturated ketones.[120] Interestingly, the two enzymes reacted regiodivergent in some cases, which allowed the selective synthesis of both ene- and enol lactones. Although the crystal structure of one of the two enzymes—BVMO from *Parvibaculum lavamentivorans*—has recently been solved, a structural explanation for its unusual reactivity has yet to be provided.[76] Only two other unsaturated ketones were reported to be accepted by BVMOs before: a substituted cyclopentenone, converted to the corresponding ene lactone by CPMO,[121] and pulegone, a cyclohexanone derivative with a double bond outside the ring on the alpha carbon, for which activity was reported with monoterpene ketone monooxygenase (MMKMO),[122] and cyclopentadecanone monooxygenase (CPDMO).[70] The three enzymes involved in campher degradation in *Pseudomonas putida*—2,5-diketocamphane 1,2-monooxygenase (2,5-DKCMO), 3,6-diketocamphane 1,6-monooxygenase (3,6-DKCMO) and OTEMO[123]—were also reported to convert several cyclopentenones and cyclohexenones, but the results were

questioned by the Alphand group.[120] Conversion of a linear α,β-unsaturated ketone to the ene ester has been shown for the Baeyer-Villiger reaction-catalyzing human FMO 5.[48] Oxidation of esters, which bear an even less electrophilic carbonyl, has been reported for a single BVMO, which is able to catalyze first the ketone oxidation and subsequently further converts the ester to its carbonate.[124]

Similarly to peroxides,[125] BVMOs were early found to promiscuously catalyze heteroatom oxidations as well.[112,126] Sulfoxidations are particularly well studied and many enzymes produced sulfoxides with high enantioselectivity.[117,127-137] Other examples include oxidations of amines,[36,116,138-139] boron,[112,140-141] and selenium.[112,142] A single report of phosphite ester and iodine oxidation yet awaits further exploration,[112] as do the few reports of epoxidations catalyzed by BVMOs.[143-144]



Scheme 3. Non-canonical oxidation reactions catalyzed by BVMOs. Solid arrows represent enzymatic catalysis, a dashed arrow indicates spontaneous reaction.

In contrast to the nucleophilic species required for the Baeyer-Villiger reaction, S-, N-, Se-, P-, and I- oxygenation require an electrophilic, protonated peroxyflavin. In line with the mechanism found for class A flavoprotein monooxygenases,[145] this hydroperoxyflavin was suggested to form in BVMOs and an apparent pK_a for the formation was determined to be 8.4 in CHMO[85] and 7.3 in PAMO.[111] However, as the protonated species in CHMO was not able to

perform sulfoxidations, the results are not fully conclusive and it was suggested that some protein conformational change is involved.[146] It was found that the enantioselectivity of heteroatom oxidation in PAMO depends on the protonation state of the peroxyflavin and the active site arginine that is crucial[91,111] for the Baeyer-Villiger reaction.[147] Results for another BVMO showed, however, that enzymatic activity does not depend on the arginine, as the mutation to alanine or glycine yielded variants with retained S- and N-oxidation activity.[148] In this scenario, the loss of arginine could have two counteracting effects: as quantum mechanics studies suggest that a nearby aspartate protonates the arginine and this stabilizes the negatively charged, deprotonated peroxyflavin,[93] arginine mutation could favor hydroperoxyflavin formation and thus the electrophilic mechanism. On the other hand, arginine loss decreases the overall reaction rate as the residue also promotes the reductive half-reaction.[111,149] Interestingly, the substitution of a highly conserved aromatic residue with arginine was found in two independent studies that screened for variants with increased sulfoxidation activity.[65,150] In most BVMOs this residue is a tryptophan that hydrogen bonds to the 3' OH of the NADP ribose. Considering the enzyme's tolerance of other aromatic residues at this position,[151] this interaction is likely not influencing the electronics at the 2' OH, which critically hydrogen bonds to the substrate carbonyl to activate it for nucleophilic attack (Scheme 2).[93] Rather, a mutation to arginine could push the positively charged coenzyme, possibly causing a disruption of the hydrogen bond to the substrate. Instead, the group might come closer to the peroxyflavin and cause its protonation; this mechanism would favor the electrophilic route and seems to be the mode of action in the closely related N-hydroxylating monooxygenases.[152]

Enzyme engineering

Besides the usefulness in gaining mechanistic insights, mutagenesis in BVMOs has been used to deliberately alter various enzyme properties. A large body of work has focused on altering substrate scope and selectivities. These studies have often focused on what have become the two prototypes, AcCHMO and PAMO. The two enzymes can be seen as the 'yin and yang' of BVMO research: AcCHMO was discovered early on, but no structure was available until very recently a 10-fold mutant was crystallized; it acts on a broad range of substrates, often shows high stereoselectivity, but is marked by poor stability. On the contrary, PAMO was discovered much later, but the crystal structure was solved immediately; its substrate scope is narrow and its stereoselectivity is often poor, but it is very stable. For these reasons mutagenesis in PAMO

focused on substrate selectivity engineering and in AcCHMO at manipulating product specificity and thermostability.

Efficient protein engineering of BVMOs became possible after recombinant strains of *E. coli*[153] and yeast[154] were available. In the absence of a crystal structure, early mutagenesis experiments focused on investigating the functional role of conserved residues.[63,155-156] In recognition of their potential for application, one of the first attempts of rational protein engineering in BVMOs was targeting their dependency on NADPH, which is more costly and less stable than NADH. By changing conserved basic residues close to the Rossmann fold, a lysine in HAPMO was identified to strongly determine NADPH specificity.[91] Mutagenesis to phenylalanine decreased the K_m for NADH ~5-fold, while mutagenesis to alanine in AcCHMO decreased it ~2-fold. A later study in PAMO did not observe the same effect upon mutating the corresponding residue, but identified a non-conserved histidine, whose mutation to glutamine decreased the K_m for NADH ~4-fold.[157] More recently a larger set of mutations was probed in AcCHMO, but the best mutant decreased the K_m only ~2.5-fold.[158] The mutations of the various studies also increased the maximal turnover rate with NADH, leading to a moderate increase in catalytic efficiencies, and decreased the specificity for NADPH (Table 2). The latter effect was especially dominant in AcCHMO when a substitution of a conserved [S/T] with glutamate was combined with targeting the previously-found lysine. The resulting mutant was still so poor with NADH, however, that bioconversions of 5 mM of AcCHMO's native substrate, cyclohexanone, was only possible when using stoichiometric amounts of the cofactor.[158] The fact that the switch of cofactor specificity—while often successful in other enzyme classes[159-160]—was largely unsuccessful in BVMOs, highlights the complex role of NADP in class B monooxygenases. It is now well known that NADP fulfills at least a dual function in catalysis (see Chapter 1). In doing so, the cofactor likely undergoes conformational changes whose stabilization and interchange need to be in a balance that is easily impaired by mutagenesis.

Table 2. Enzyme variants generated to switch cofactor specificity.

enzyme	mutation(s)	fold increase $k_{cat,NADH}$	fold decrease $K_{m,NADH}$	fold increase $k_{cat,NADH}/K_{m,NADH}$	fold decrease NADPH/NADH	Ref
HAPMO	K439F	1.4	4.8	6.7	410	[91]
PAMO	H220Q	6.9	3.7	3.3	8.6	[157]
AcCHMO	K326A	0.4	1.8	0.7	58	[91]
AcCHMO	S186P/S208E/ K326H	3.1	2.5	8	1900	[158]

An even more important factor for application is catalyst stability. For many enzymes, the main focus of attention is operational stability, as storage stability is more easily addressed—most enzymes can be kept frozen in solution for months or years, or otherwise kept as lyophilized powders. For BVMOs, one study found that lyophilization in the presence of sucrose aids in preserving catalytic activity.[161] In the course of this work, the generally very poor stability of AcCHMO was also quantified: upon storage at 4 °C, the enzyme lost half of its activity after 72 h. Being a well-known phenomenon, it has been tried to overcome the issue of instability by enzyme engineering several times; a task that was complicated by the absence of a crystal structure. Another issue is a lack of agreement in literature about how thermostability best is measured and compared. While most agree that the aim is to improve the stability over time under process conditions, it is a non-trivial parameter to measure in a high-throughput fashion. As a popular alternative, it has emerged to initially screen the proteins' melting temperature (T_m), which is defined as the midpoint of a melt curve reflecting the unfolding of a protein ensemble.[162-163] Various methods using common laboratory equipment allow the quick measurement of this parameter, requiring usually very small amounts of sample and often in high-throughput. For BVMOs, a method exploiting flavin fluorescence termed ThermoFAD allows T_m determination without the usually required addition of dyes.[162] Nevertheless, the technique is not always applied, and a comparison of literature is not easy as most studies measure stability under different conditions (among other, enzyme formulation and temperature).

The first report of a more stable AcCHMO mutant targeted the oxidative stability of the enzyme, rationalizing that the hydrogen peroxide side product could inactivate the enzyme through oxidation of sulfur-containing residues.[164] By mutating all cysteines and methionines to amino acids found in homologous BVMOs, several positions were identified to increase substrate conversions in the presence of hydrogen peroxide and at elevated temperatures. The best variants of the subsequently generated combinatorial mutants showed a strongly increased hydrogen peroxide tolerance, and a 7 °C upshift of the temperature at which 50% of activity remained. With the aim of increasing the thermal stability of AcCHMO, two parallel studies later created a homology model of the enzyme and used computational prediction to design stabilizing disulfide bridges. The first study reported an increase in T_m of 6 °C and a >10-fold increase in half-life at 37 °C for the best mutant, which interestingly was a disulfide bridge that span only a single residue.[165] Combining several disulfide bridges led to strongly reduced expression levels, however. The second study tested four disulfide bridge designs, and found an increase in T_m of 5 °C for the best variant.[166] Upon finding that the stabilization occurs although the disulfide

bridge does not form in solution, the individual mutations were tested and the effect thus traced to a single threonine to cysteine exchange. This variant had a 6 °C higher T_m, and a ~15-fold increase in half-life. Stabilization upon cysteine introduction is a surprising result, seemingly in contradiction to the earlier study that aimed to remove sulfur-containing residues. Although no clear explanation exists, the oxidation by hydrogen peroxide in this particular area of the protein may not negatively affect protein stability and act as a scavenger of reactive oxygen species.

As none of these engineering efforts managed to develop a variant that reaches the stability levels of PAMO, which has a T_m of 61 °C[167] and does not lose activity for several days when stored at room temperature,[168] alternative strategies used PAMO as the engineering scaffold. Where the long-studied AcCHMO's catalytic properties were often found to be excellent, PAMO mostly proved to be a relatively poor catalyst for synthetically interesting reactions. The biggest weakness was the limitation of the substrate scope to small aromatic ketones, and PAMO's inactivity on cyclohexanone, which prevents an application in biotechnological nylon production.[168] However, engineering of PAMO could finally be based on rational considerations, since the enzyme was crystallized right after its discovery and this represented the first structure of a BVMO.[64] In five separate studies, the group of Reetz aimed at engineering PAMO toward activity with cyclohexanone and its derivatives. Upon noticing that PAMO differed from CHMOs by a two residue insertion in an active-site loop, this so-called 'bulge' was deleted in the first PAMO engineering study[169] and subjected to random mutagenesis in the second.[170] Although it was shown that either deletion or mutation increased PAMO's activity on cyclic ketones, the generated mutants were still limited to substrates containing the phenyl moiety. When the randomized region was then expanded to the entire active site, the only mutations that emerged were again the ones that targeted the bulge.[171] Therefore the authors decided to change strategy and targeted two conserved proline residues in the vicinity of, but not directly shaping the active site. Substitution of the proline directly adjacent to the bulge turned out to strongly increase enzymatic activity with a range of 2-substituted cyclohexanone derivatives that did not need to contain the phenyl group.[171] Concluding that successful proline mutagenesis may act by influencing conformational changes involved in catalysis, the authors expended their investigation towards a proline found behind a loop they assumed to be critical for domain interaction.[172] Mutagenesis of this residue and a neighboring glutamate again increased the activity with cyclic substituted cyclohexanones. With these residues being far away from the active site, the authors suggested that they induced an allosteric effect that enables domain movements favoring

the catalytic activity. In their most recent study, the authors eventually combined all the previous hotspots and randomized the bulge residues, while fixing the mutations of the two proline residues and one neighbor.[173] This final mutant that contained two additional substitutions on the bulge showed for the first time an activity with cyclohexanone, although the low rate (k_{cat} = 0.3 s^{-1}) only allowed the conversion of 2 mM. Most recently, a different group achieved conversions of 10 mM of cyclohexanone by combining the mutation of the conserved proline with a mutation of the active-site isoleucine identified as a hot spot by the Fraaije group.[174] This residue emerged in a study in which they designed mutations on the basis of a structural comparisons with a model of another promiscuous BVMO, cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 (CPMO). Using site-directed mutagenesis, 15 PAMO residues were mutated in order to map out crucial residues in the active site.[175] In another report they identified an active-site methionine that improved the activity with aromatic compounds and increased the heteroatom oxidation activity.[116] Interestingly, this mutant was able to produce indigo by converting indole through an apparent N-oxidation mechanism. In a structural study, PAMO was then also crystalized with a substrate analog in the active site, thus further narrowing down the residues important for substrate binding.[75] With this combined insight, 11 residues were then chosen for simultaneous randomization.[176] A screen for enzymatic activity on cyclopentanone and cyclohexanone was conducted for 1500 clones, which still represented only a fraction of the statistically possible mutant combinations, however. A single clone containing four substitutions was identified in this screen that had activity on cyclopentanone. One mutation targeted a bulge-adjacent residue that also emerged in the Reetz libraries,[173] and three mutations occurred in residues located in the tunnel leading toward the active site. The biochemical characterization of this mutant showed that it had a strongly expanded substrate scope and accepted various aliphatic ketones. When a recent study also found indications for an important role of the substrate tunnel by identifying in it a stable binding site for ligands, a drastic engineering attempt was conducted: to establish whether the tunnel might be the true determinant of substrate specificity, two mutants were created, which switched the entire tunnel (25 mutations) or the tunnel and the active site (38 mutations) for the residues found in a CHMO (Chapter 8).[72] This attempt turned out to be unsuccessful, however—although the mutants could be produced and bound FAD, no catalytic activity with a range of substrates was found. A similarly drastic approach was conducted in a study employing subdomain shuffling, which resulted in the creation of enzyme chimera.[167] Exchange of PAMO's C-terminus, which harbors the active site bulge and a large, mobile loop

suspected to influence catalysis (Chapter 1)[177] resulted in variants with altered, but mostly PAMO-like activity.

Domain movement may indeed play a more important part in substrate acceptance than anticipated so far—in AcCHMO, mutations in the hinge region connecting the FAD and NADP domain had a profound effect on catalytic activities.[178] Since the enzyme is already naturally promiscuous—with the reported substrates going to into the hundreds[179-181]—only few other studies aimed at altering its substrate scope. With CHMO's main limit being substrate size, these efforts were often with a commercial interest, aiming to generate highly evolved variants optimized for a specific bulky target—commonly pharmaceuticals. A prominent example was the development of mutants with high sulfoxidation activity on the precursor of esomeprazole.[65,182] Conversion of steroids is also of potential pharmaceutical interest, but engineering of CHMO seems unnecessary as there are several BVMOs available that naturally accept them as substrates.[59,183-184] In one report, novel activities on exo tricyclic ketones was discovered for mutants originally evolved to switch product specificty,[185] which in fact is the most common engineering aim in CHMOs.

As oxygen insertion can occur on either side of the carbonyl group, the Baeyer-Villiger reaction can afford two regioisomeric products. As specified before, the outcome is dictated by various effects, which lead to a predictable bond migration in chemical transformations, while often resulting in the non-canonical products by enzyme catalysis. In this case, the resulting ester has been referred to as "abnormal", while canonical bond migration affords the "normal" ester. In the case of cyclic substituted ketones, it was suggested to avoid ambiguity by using the terms "distal" and "proximal" lactones instead.[186-187] Although originally proposed for cyclic ketones with a substituent on the alpha carbon,[186] it has become more common for ketones with substituents further from the carbonyl.[188] BVMO-catalyzed reactions are also often stereoselective, and regio- and stereoisomerism is often intimately connected in Baeyer-Villiger reactions. Considering prochiral 4-substituted cyclohexanones, for example, where no stereo control can distinguish substrate enantiomers, the side of oxygen insertion (i.e. regioselectivity) still determines the product enantiomers (Chapter 9).[189] Another interesting point is the often observed regiodivergent conversion of chiral racemic ketones, where the regioselectivity of the reaction differs for each enantiomer. This effect has frequently been observed to yield both regioisomers in a reaction, but where each is produced enantiomerically pure or enriched. This behavior can be assessed with substrates such as *rac*-bicyclo[3.2.0]hept-2-en-6-one. This bicyclic ketone has become a model substrate[190] since it was first used to demonstrate BVMO-mediated asymmetric synthesis;[191] and an industrial,

BVMO-catalyzed process has been established.[192] Being of rather unpredictable outcome, the specificity of a large number of BVMO variants with countless substrates have been collected in extensive reviews.[179-180,188,193] Before the availability of crystal structures, the site-specificity of BVMOs has been the subject to controversial debate, and it has been tried to use in mapping the active site of AcCHMO based on the selectivity with various structurally restrained substrates.[194-195] While the models were still refined after the PAMO structure was available,[196] the subsequent RhCHMO structures and technological development led to an increased use of computational methods. Considering the complexity of the reaction mechanism and the partial uncertainty in enzyme catalysis, however, it is maybe no surprise that most protein engineering studies still largely rely on random or semi-random libraries and use computational tools analytically rather than predictively.

A case of more targeted engineering was a BVMO involved in the *Streptomyces arenae* biosynthesis of pentalenolactone D.[197] While this antibiotic features an abnormal lactone moiety, it was found that a homologous strain produces the metabolite as the normal isomer.[198] A few differing residues were identified by sequence alignment of the responsible homologous BVMOs and a single amino acid exchange was sufficient to completely inverse the selectivity of the abnormal lactone-forming enzyme. The opposite mutation in the natively normal lactone-forming enzyme did not cause abnormal product formation, however, and the enzyme was moreover largely expressed in inclusion bodies. The unique ability to deliberately produce abnormal esters is one of the synthetically most interesting feature of BVMOs. Yet, also most other regioselectivity engineering studies reported a switch of selectivity toward the normal ester. The prevalence of this kind of "demolishing" is likely because abnormal migration needs to be strictly enforced by the active site through steric control, while normal migration occurs also in the absence of a strict restraint via electronic control. Besides following the logic that the flavin intermediate underlies the same chemical principles as any other reactant, this notion was also substantiated by combined QM/MM studies[70,151] and an unusual experimental approach: upon cumulatively removing all active-site residues involved in substrate-binding, it was observed that the regioselectivities of a CHMO approached the ratios obtained with chemical catalysts.[72] A popular target molecule has been the terpene *trans*-dihydrocarvone, a 2- and 5-substituted cyclohexanone derivative with two chiral centers that many BVMOs can convert with high selectivity.[199] In a traditional alanine-scanning mutagenesis experiment for the active-site residues of a CHMO, the Bornscheuer group already noted that exchange of one particular residue led to a switch in regioselectivity from fully abnormal to

mostly normal lactone.[200] After two additional alanine mutations were introduced based on the alanine-scanning result and a docking experiment, the resulting mutant produced exclusively the normal lactone. Interestingly, all targeted residues were phenylalanines. The authors successfully transferred the mutations to AcCHMO, where the effect prevailed. When they later probed the mutations in OTEMO, they discovered that a single substitution—corresponding to the mutation showing the strongest effect in CHMO—was sufficient to invert regioselectivity in OTEMO.[201] The results were subsequently rationalized in a study from Scrutton and coworkers, who introduced the same mutations in another CHMO, solved the crystal structure of the mutant, and performed computational analyses.[70] As expected from three phenylalanine to alanine mutations, it appears that the mutations removed steric restraints exhibited by the wild type, thus inducing the reaction fate to be determined by the lower energy barrier associated with normal lactone production. Although the mutations proved mostly transferable among enzyme variants, they did not exhibit the same effect on other substrates. For example, the triple alanine mutant produced only slightly more than the 50% of normal lactone seen in conversions of (−)-bicyclo[3.2.0]hept-2-en-6-one with OTEMO, while a double valine substitutions achieved 95% normal lactone.[201] Surprisingly, the authors could not reproduce the result when using purified enzymes instead of whole cells, although they could partially restore selectivity by adding FAD to the purification buffer. This effect was, however, not observed for another mutant, already known earlier to influence selectivity,[68] in which a conserved tryptophan to alanine mutation caused 95% abnormal lactone production. Mutagenesis-induced activity increase towards abnormal ester—the more interesting, though, more challenging task—is commonly only observed sporadically. Targeted engineering of abnormal product formation has been attempted in several studies for 2-butanone. The reaction is of synthetic interest, as the abnormal product, methyl propanoate, can be converted to methyl methacrylate, an acrylic plastic produced industrially on megaton scale annually.[202] An initial screen of several BVMOs showed moderate activities with most BVMOs, and the best enzyme, AcCHMO, produced approximately 25% abnormal product.[203] A small library based on structure-inspired rational design was then tested for improvement, and a double mutation identified, which increased the yield and produced 43% of the abnormal product.[204] The fact that a full switch was not achieved reflects the apparent difficulty in engineering a preferred migration of the least favored substituent, the methyl group. Recently, this was achieved with even more demanding substrates, in which the methyl group had to compete with phenyl

substituents by screening larger libraries and performing directed evolution (Chapter 9).[151]

Literature examples of stereoselectivity engineering are more frequent and have been reviewed previously.[188,205] Many beneficial mutations have been identified by random mutagenesis, and a successful technique to reduce screening effort was the creation of focused libraries that target residues close to the active site.[16,206] Computational analyses have become popular, but the methods yet have to be proven reliable enough to exhibit predictive power.

Concluding remarks

Biotechnology is at an exciting crossroads where ever more discoveries lead to the developments of applications in the various sub-disciplines that have (e)merged. Biocatalysis is maturing to a serious alternative to classical chemical transformations and this hopefully can contribute to a greener industry and new products at the same time. Baeyer-Villiger monooxygenases are intriguing catalysts for a demanding reaction that allow unrivaled control of the reaction selectivity. Numerous variants have been described that feature activities suitable for countless reactions of synthetic value. Limitations, such as cofactor dependency, limited stability and undesired specificities are clearly defined and active research is making progress in overcoming these. A suitable tool to that end is enzyme engineering, and directed evolution has been most effective in altering undesired enzyme properties. Computational design has a great potential, but needs to become more reliable—a task that will not least be facilitated by unravelling the last remaining mechanistic open questions of BVMO catalysis.

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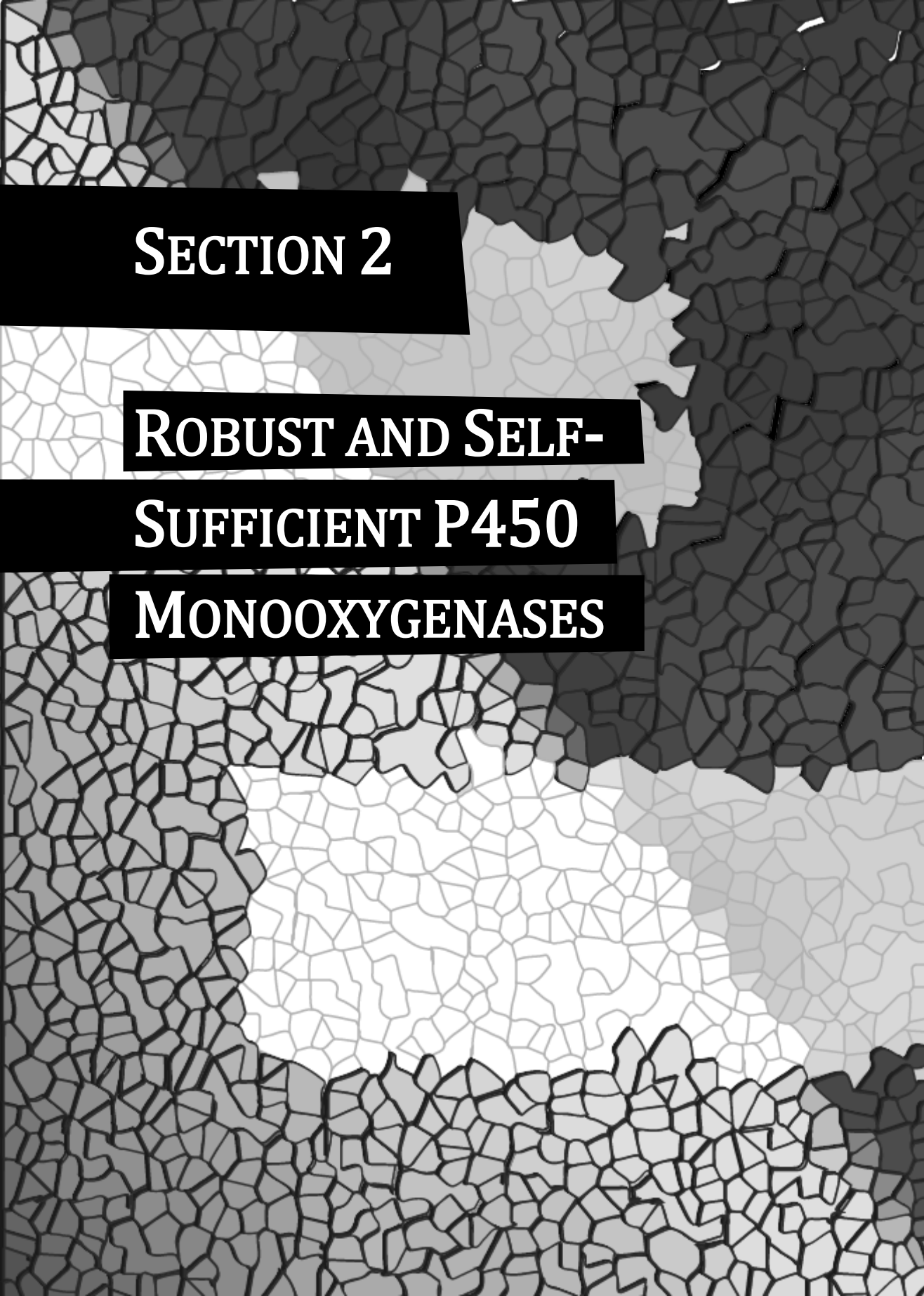

Section 2

Robust and Self-Sufficient P450 Monooxygenases